Investigation of the susceptibility of various strains of mice to methyllycaconitine toxicosis[1]

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ABSTRACT: Although the mechanism of action for larkspur alkaloids has been described, little information is available on the variation of the physiological response of individual animals to larkspur alkaloids. Anecdotal observations and pilot studies in cattle indicate that there is animal-to-animal variation in response to a debilitating dose of larkspur alkaloids. The objective of this study was to determine whether there is variation in susceptibility of different strains of mice to larkspur alkaloid toxicosis and to identify factors responsible for the variation that could then be used as a model for studies in cattle. The acute toxicity of methyllycaconitine (MLA) in 9 different inbred strains of mice was compared. The rank order, from most to least susceptible, was A/J > B10 > FVB > BALB/c > C57Bl/6 > NZW > C3H > DBA >129. The calculated LD_{50} ranged from 3.3 ± 0.2 to 5.8 ± 0.8 mg/kg of BW. The toxicokinetic profiles of MLA in the susceptible A/J strain and the more resistant 129 strain were compared to determine whether their differences in susceptibility were due to differences in their ability to eliminate MLA. The differences in toxicokinetic variables observed did not explain the differences in susceptibility. The protein expression of various nicotinic acetylcholine receptor (nAChR) subunits was also compared between the more resistant 129 strain and the susceptible A/J strain. The 129 strain of mice had twice the amount of α7 nAChR subunit expression as the A/J strain, which was in direct proportion to the approximately 2-fold difference in LD_{50}. There was also a significant difference ($P < 0.05$) in expression of the α3 and α5 nAChR subunits between the 129 and A/J strains, with the 129 strain having a greater expression in each case. These data suggest that the increased susceptibility of the A/J mice could be due to a reduced expression of nAChR subunits. Similar analyses need to be made in cattle to determine whether there is a difference between breeds in susceptibility to larkspur poisoning and to identify the factors that regulate their susceptibility to larkspur poisoning. This information would be useful for livestock producers in their breeding, culling, and grazing management programs to reduce or prevent larkspur poisoning on rangelands.

Key words: *Delphinium*, larkspur, lethal dose 50%, methyllycaconitine, mouse strain

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INTRODUCTION

Larkspur (*Delphinium* spp.) is a toxic plant located in the foothill and mountain rangelands of the western United States (Pfister et al., 1999). The toxicity of larkspur plants is due to the more than 18 norditerpenoid alkaloids, each with varying degrees of affinity, and potency at nicotinic acetylcholine receptors (**nAChR**; Macallan et al., 1988; Dobelis et al., 1999). Previous research has demonstrated that the physiological effects of methyllycaconitine (**MLA**), one of the more abundant toxic alkaloids in larkspur, is attributable to its high affinity to nAChR in muscle and nervous systems (Benn and Jacyno, 1983; Stegelmeier et al., 1998). Methyllycaconitine has been shown to be a potent and selective competitive antagonist with nanomolar affinity at α7 nAChR and micromolar affinity at other nAChR (Ward et al., 1990; Alkondon et al., 1992; Lopez et al., 1998; Daly, 2005).

Although the mechanism of action for larkspur alkaloids has been described, little information is available on the variation in animal responses to larkspur alkaloids. Anecdotal observations and pilot studies in cattle (Green et al., 2009) indicated that there is variation in response to a debilitating dose of larkspur. The susceptibility of cattle to larkspur alkaloids acting at nAChR may be due to genetic differences, which cause changes in nAChR number or function. It has been well

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documented in mice that the physiological response to nicotinic agonists varies between mouse strains, and that genetic differences in nAChR subunits in those strains can be correlated with functional changes in the response to nAChR agonists such as nicotine (Collins et al., 1988; Marks et al., 1989; Miner and Collins, 1989; Crawley et al., 1997; Dobelis et al., 2002; Mexal et al., 2007). The objective of this study was to determine whether there is variation in the susceptibility of different strains of mice to MLA and to identify factors responsible for the variation that could be used as a model for cattle.

MATERIALS AND METHODS

All procedures were conducted under veterinary supervision and were approved by the Utah State University Animal Care and Use Committee.

Alkaloid Preparation

The MLA used in this study was extracted from *Delphinium barbeyi* (Huth) Huth following previously published methods (Pelletier et al., 1981, 1989; Manners et al., 1991). The purified MLA was suspended in physiological buffered saline solution, and the pH was reduced with HCl to achieve solubility. Ammonium hydroxide was then added to the solution to increase the pH to as close to physiological pH as possible (pH 6.0) while still retaining solubility. The MLA solution (0.8 mg/mL) was stored in a sterile injection vial at 4°C until use. No adverse effects were seen after injections (0.05 to 0.2 mL) of a solution with this pH (pH 6.0).

Animals

Nine inbred strains (A/J, B10.D2-H2oSNJ, FVB/NJ, BALB/cJ, C57BL/6J, NZW/LaCJ, C3H/HeJ, DBA/2J, 129/SvImJ) of male mice were purchased from Jackson Laboratories (Bar Harbor, ME). Mice were acclimated for 3 to 4 d with free access to food and water before beginning the experiments.

Median Lethal Dose Determination

The LD_{50} of MLA in 9 different strains (A/J, B10.D2-H2oSNJ, FVB/NJ, BALB/cJ, C57BL/6J, NZW/LaCJ, C3H/HeJ, DBA/2J, 129/SvImJ) was determined by using male mice weighing 21 ± 4 g. Between 0.05 and 0.2 mL of MLA, in buffered saline, was injected via the tail vein. Mice were observed for clinical effects and mortality, and the LD_{50} of MLA in each strain of mice was determined by using a modified up-and-down method (Bruce, 1987). In this method of acute toxicity testing, animals are dosed one at a time. If the first animal survives, then the next animal receives a larger dose, whereas if the first animal dies, the next animal receives a smaller dose. The dose for each successive animal is adjusted up or down depending on the outcome from the previous animal. This causes the doses to be adjusted rapidly toward the LD_{50} and then to be maintained in the region of the LD_{50}. This method is preferred because fewer animals are required; however, it results in unbalanced numbers in each group. The LD_{50} values were calculated by using PROC PROBIT in a logistic regression (SAS Inst. Inc., Cary, NC).

Toxicokinetic Analyses

For toxicokinetic studies, 35 A/J strain and 35 129/SvlmJ strain mice weighing 19 ± 2 g were randomly divided into 7 groups to obtain samples at 7 time points after injection (1, 2, 5, 10, 15, 30, and 60 min) for each strain. Mice were dosed via the tail vein with 70% of the LD_{50} for each strain, as determined in the LD_{50} studies. This dose has been shown to produce clinical signs but not to be lethal (Stegelmeier et al., 2003). Mice were killed by CO_2 asphyxiation, followed by cervical dislocation. Brain, liver, kidney, skeletal muscle from the biceps femoris of the pelvic limb, and serum were collected and frozen at −20°C until analyzed.

Sample Extractions

Samples (from approximately 0.2 g of tissue or 1 mL of serum) were prepared as described previously (Stegelmeier et al., 2003) and stored at −20°C until analysis by HPLC-mass spectrometry. This method of extraction has been shown to have a recovery rate of approximately 95% and to have approximately 11% variation.

Sample and Standard Preparation

Samples were resuspended in 1.0 mL of methanol:20 m*M* ammonium acetate (50:50, vol/vol) and mixed for 1 min with a Vortex Genie, followed by filtration through a 0.2-μm nylon syringe filter into HPLC autosampler vials. Calibration standards of MLA were prepared in ethanol from stock solutions (1.0 mg/mL) stored at −20°C. Diluted standards were prepared fresh daily to 1000, 500, 250, 125, 62.5, and 31.2 ng/mL. Standards were analyzed after every 40 samples.

Mass Spectrometry Analysis

Analysis of MLA in the samples was accomplished by using a Surveyor HPLC and autosampler system coupled to a ThermoFinnigan LCQ Advantage Max mass spectrometer (ThermoFinnigan, San Jose, CA) with modification of the methods described previously (Turek et al., 1995; Gardner et al., 1999; Stegelmeier et al., 2003). The instrument parameters were maximized for detection of the alkaloid reserpine by using the autotune feature. Samples (25 μL) were injected with a Surveyor autosampler onto a Betasil C18 HPLC column (100 × 2 mm, 5 μm, 100 A, Keystone Scientific, Bellefonte, PA). The column was eluted by using a gradient

flow of methanol (A):20 m*M* ammonium acetate (B), beginning with 65% A and a linear gradient to 80% A from 0 to 6 min, 80% A from 6 to 7 min, and equilibration at 65% A from 7 to 12 min. Flow rate was 0.3 mL/min. Retention time for MLA under these conditions was approximately 3.7 min. Flow from the HPLC was connected directly to the electrospray source of the mass spectrometer. The mass spectrometer was operated in a full-scan MS-MS mode after fragmentation of the selected parent ion of 683.3 (MH^+, for MLA). Selected ion chromatograms for m/z 665.3 were used for detection and quantification of MLA.

nAchR Immunoblot Analysis

Sections of brain tissues were homogenized in 100 m*M* PBS, pH 7.4, containing 1 m*M* EDTA, 250 m*M* sucrose, and a protease inhibitor cocktail (Complete, Roche Applied Science, Indianapolis, IN). Equivalent amounts of protein were then diluted in sample buffer under reducing conditions (125 m*M* Tris-HCl, pH 6.8, 0.5% SDS, 20% glycerol, 40 m*M* dithiothreitol, and bromophenol blue), boiled, and resolved on 10% acrylamide gels. After transfer to nitrocellulose (Bio-Rad, Hercules, CA), nonspecific binding was blocked with 5% nonfat dried milk, and blots were probed with a goat anti-nAchR α3 antibody (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-nAChR α5 antibody (1:500; Santa Cruz Biotechnology), or rabbit anti-nAChR α7 antibody (1:500; Chemicon International, Temecula, CA), followed by incubation with an appropriate peroxidase-conjugated secondary antibody (mouse anti-goat IgG horseradish peroxidase, 1:2000, Chemicon International; and goat anti-rabbit IgG horseradish peroxidase, 1:2000, Millipore, Temecula, CA). Protein signals were visualized by chemiluminescent detection (ECL, Amersham Pharmacia Biotech, Piscataway, NJ). Exposed X-ray films or direct chemiluminescent detection from nitrocellulose membranes were scanned and analyzed with a Kodak Image Station 2000RT imager and its software (Eastman Kodak, Rochester, NY).

Analysis and Statistics

Confidence (fiducial) intervals (95%) were calculated for LD_{50} values by using logistic regression. Statistical comparisons of toxicokinetic profiles between groups were performed by ANOVA with a post hoc test of significance between individual groups. Differences were considered significant at $P < 0.05$. The alkaloid concentrations were plotted by using SigmaPlot (SPSS Inc., Richmond, CA). Kinetic profiles were analyzed by using standard pharmacokinetic software (PK Solutions 2.0 for Noncompartmental Pharmacokinetic Data Analysis, Summit Research Services, Montrose, CO). A curve-stripping procedure was used to determine the basic pharmacokinetic variables of half-life, and rate for the elimination phase of the MLA concentration curve. The following variables were determined: $t_{1/2} = 0.693/k_{elim}$, C_{max}, T_{max}, AUC. The $t_{1/2}$ is the elimination half-life, k_{elim} is the elimination rate, and C_{max} and T_{max} describe the concentration and time of maximal alkaloid concentrations. A trapezoidal method was used to determine the area under the curve (**AUC**) of a concentration vs. time graph.

RESULTS

The acute toxicity of MLA in 9 different inbred strains of mice was compared. Inbred strains of mice were used in this study in an attempt to minimize intrastrain differences, and were selected based on their genetic diversity. The calculated LD_{50} ranged from 3.3 ± 0.2 to 5.8 ± 0.8 mg/kg of BW (Figure 1 and Table 1). The A/J strain of mice was found to be the most susceptible to the acute toxicity of MLA, with a calculated LD_{50} of 3.3 ± 0.2 mg/kg of BW. The B10 strain was also found to be susceptible, with an LD_{50} of 3.5 ± 0.01 mg/kg of BW. The 129 strain was found to be the most resistant to the acute toxicity of MLA, with an LD_{50} of 5.8 ± 0.8 mg/kg of BW. The DBA strain was also found to be more resistant, with an LD_{50} of 5.0 ± 0.2 mg/kg of BW. The FVB, BALB/c, C57Bl/6, NZW, and C3H strains all had intermediate susceptibility to the acute toxicity of MLA.

Clinical signs of toxicity were similar in all strains of mice and were similar to those described previously (Stegelmeier et al., 2003). Within seconds of injection, mice were reluctant to move, and they sat hunched with diffuse piloerection, resulting in a scruffy appearance. Within 1 min, mice developed muscle tremors and spastic jerky muscular convulsions. These jerky convulsions were followed by dyspnea, which caused the nose and toes to become cyanotic. Typically, animals died within 2 min of treatment. Animals that did not die seemed to have recovered and were completely normal within 20 min. There were no differences in the time to death among any of the strains.

The toxicokinetic profiles of MLA in both the susceptible A/J and more resistant 129 strains of mice were determined (Figure 2 and Table 2). Elimination profiles of MLA in the liver, kidney, brain, and muscle tissues, as well as in serum, were compared in the 129 mice and the A/J mice to determine whether the differences in susceptibility could be attributed to differences in concentration of MLA in these tissues. There were no differences ($P > 0.05$) in the $t_{1/2}$ of MLA from the liver, kidney, muscle, or serum between the 2 strains (Table 2). There was, however, a difference ($P < 0.05$) in the $t_{1/2}$ of MLA from the brain, with the A/J mice having a $t_{1/2}$ of approximately 12 min compared with 22.5 min for the 129 strain. This indicated that the susceptible A/J mice were capable of eliminating MLA more efficiently from the brain than were the more resistant 129 mice. There was a difference ($P < 0.05$) in the C_{max} for

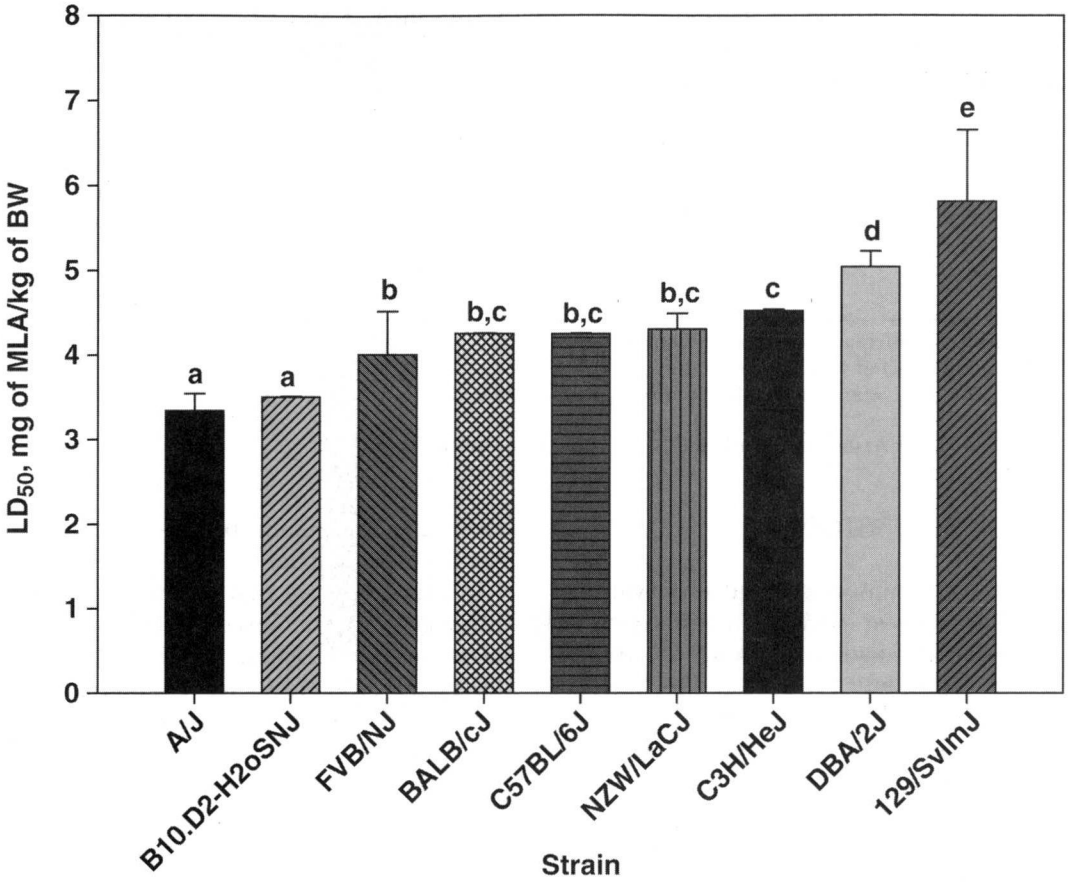

Figure 1. Comparison of various strains of mice for acute toxicity of methyllycaconitine (MLA). The data represent the LD_{50} for 9 different inbred strains of mice. Results represent the mean ± SD of 10 to 40 mice per strain. [a–e]Strains with different letters were significantly different ($P < 0.05$).

only kidney tissue, with the 129 strain having a C_{max} of 3.7 ± 0.7 μg of MLA/g of tissue vs. 2.7 ± 0.2 μg/g for the A/J strain. No differences in T_{max} were observed ($P > 0.05$). Differences ($P < 0.05$) were observed in the AUC, or total amount of bioavailable alkaloid, in the serum (Figure 2), brain, and kidney. However, the more resistant 129 strain was found to have the greater concentration of MLA in these tissues.

The protein expression of various nAChR subunits was compared between the 129 and A/J strains (Figure 3). The more resistant 129 strain of mice had 2 times the amount of α7 nAChR subunit expression as the susceptible A/J strain, which was in direct proportion to the approximately 2-fold difference in LD_{50}. There was also a significant difference ($P < 0.05$) in expression of the α3 and α5 nAChR subunits between the 129 and A/J strains, with the 129 strain having a greater expression in each case. These data suggest that the increased susceptibility of the A/J mice could be due to less expression of nAChR subunits.

Table 1. Susceptibility of various strains of mice to the acute toxicity of methyllycaconitine (MLA)

Strain	Sample size	LD_{50},[1] mg/kg	BW,[2] g	Injection volume,[3] mL
A/J	37	3.34 ± 0.20[a]	21.9 ± 1.9	0.09 ± 0.01
B10.D2-H2oSNJ	10	3.50 ± 0.01[a]	18.9 ± 1.6	0.08 ± 0.01
FVB/NJ	40	4.00 ± 0.51[b]	24.9 ± 1.6	0.12 ± 0.02
BALB/cJ	11	4.25 ± 0.01[bc]	19.7 ± 1.8	0.11 ± 0.01
C57BL/6J	11	4.25 ± 0.01[bc]	19.3 ± 1.5	0.10 ± 0.01
NZW/LaCJ	15	4.30 ± 0.19[bc]	25.9 ± 1.9	0.14 ± 0.01
C3H/HeJ	12	4.52 ± 0.02[c]	20.9 ± 1.5	0.11 ± 0.02
DBA/2J	34	5.04 ± 0.18[d]	21.0 ± 4.8	0.13 ± 0.03
129/SvImJ	36	5.81 ± 0.84[e]	21.4 ± 3.3	0.15 ± 0.02

[a–e]Strains with different superscript letters were significantly different ($P < 0.05$).
[1]Results represent the mean ± SD of the LD_{50}.
[2]Results represent the mean ± SD of the animal BW.
[3]Results represent the mean ± SD of the injection volume of a 0.8-mg/mL solution of MLA.

Table 2. The toxicokinetic profiles of methyllycaconitine (MLA) in A/J and 129 strains of mice[1]

Variable[2]	Strain	Liver	Kidney	Brain	Muscle	Serum[3]
$t_{1/2}$, min	A/J	20.2 ± 8.7	10.6 ± 1.5	12.0 ± 2.5[a]	12.9 ± 2.2	9.5 ± 4.4
	129	11.3 ± 4.7	9.8 ± 0.9	22.5 ± 3.8	13.2 ± 4.7	8.4 ± 2.9
C_{max}, μg/g	A/J	0.8 ± 0.5	2.7 ± 0.2[a]	0.06 ± 0.03	0.4 ± 0.1	0.4 ± 0.1
	129	1.0 ± 0.2	3.7 ± 0.7	0.06 ± 0.01	0.5 ± 0.1	0.7 ± 0.2
T_{max}, min	A/J	1.3 ± 0.5	1.2 ± 0.4	1.4 ± 0.5	2.0 ± 0.0	1.4 ± 0.5
	129	1.2 ± 0.4	1.4 ± 0.5	1.6 ± 0.5	2.0 ± 0.0	1.2 ± 0.8
AUC, μg-min/g	A/J	6.3 ± 1.6	24.2 ± 3.2[a]	0.7 ± 0.0[a]	3.3 ± 0.5	4.8 ± 0.8[a]
	129	5.3 ± 2.1	31.7 ± 1.5	1.3 ± 0.2	4.3 ± 1.1	6.8 ± 1.2

[a]Within a variable, significant difference between the 2 strains ($P < 0.05$).

[1]Results represent the mean ± SD of 4 to 5 mice per group.

[2]$t_{1/2}$ = elimination half-life; AUC = area under the curve. C_{max} and T_{max} describe the maximal concentrations and time of maximal MLA concentrations, respectively.

[3]The units for C_{max} and AUC for serum are μg/mL and μg-min/mL, respectively.

DISCUSSION

Over the past several years, anecdotal observations have been made that suggest there is a difference in susceptibility to larkspur poisoning among the different breeds of cattle commonly grazed on the western US rangelands. Until recently, this potential difference in breeds had not been studied in a research setting. Preliminary data by Green et al. (2009), performed in a controlled research setting, suggest there is indeed a difference in the susceptibility of different breeds of cattle to larkspur toxicity. However, before engaging in a large study using cattle to identify potential genetic differences, we wanted to perform similar studies in a rodent model, with the hypothesis that differences discovered in mice could be used as candidate markers for studies using cattle.

In this study, we first determined whether there were differences in the susceptibility of different strains of mice to acute larkspur toxicity. To accomplish this, we determined the LD_{50} for 9 different inbred strains of mice to a bolus dose of purified MLA administered i.v. There was a difference of approximately 2-fold in the LD_{50} in the strains studied. The A/J and 129 strains were selected as representative strains for a susceptible and more resistant strain, respectively. These 2 strains were further characterized to identify factors regulating the susceptibility to acute larkspur toxicity.

The toxicokinetic profiles of MLA in the A/J and 129 strains were compared to determine whether their

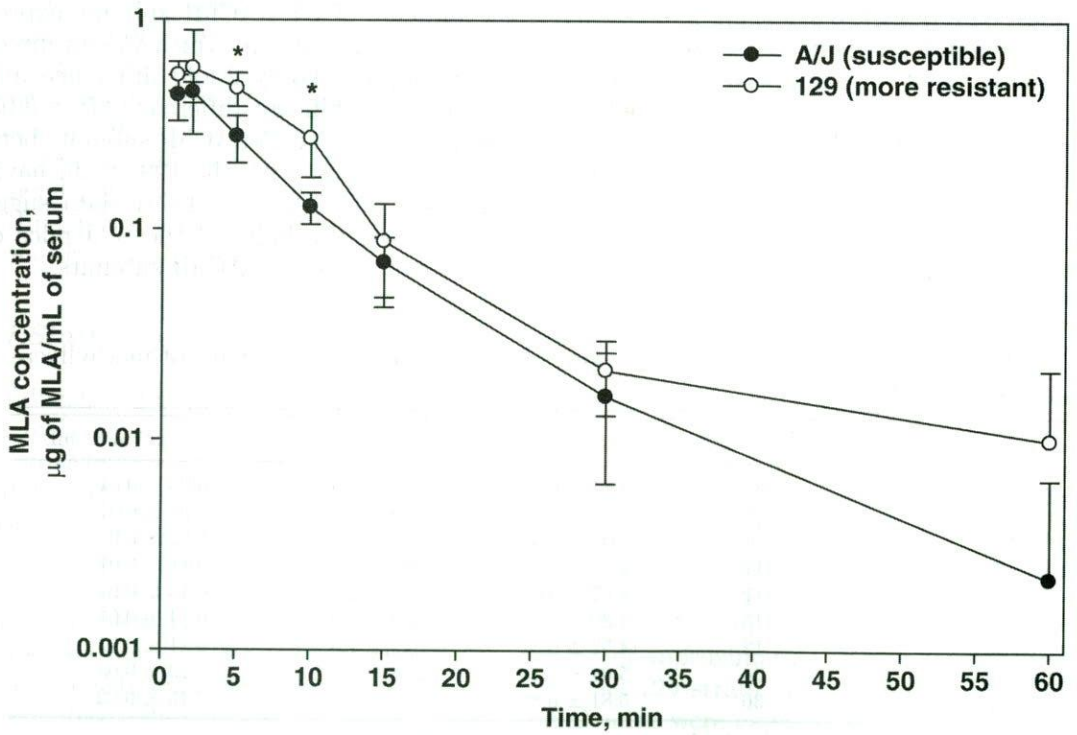

Figure 2. The toxicokinetic profile of methyllycaconitine (MLA) in serum from the susceptible A/J strain and more resistant 129 strain of mice. The data represent the serum MLA concentrations plotted as a semilogarithmic graph. Results represent the mean ± SD of 4 to 5 mice per strain per time point; *$P < 0.05$ as compared with the A/J strain.

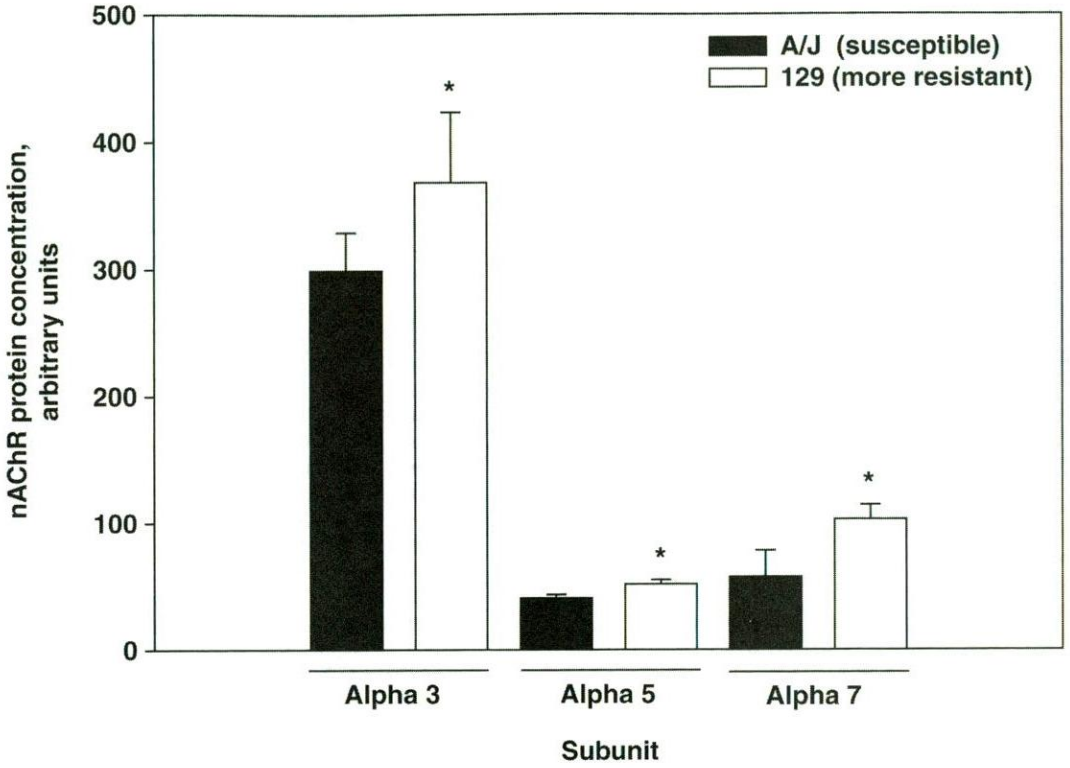

Figure 3. Brain protein expression of nicotinic acetylcholine receptor (nAChR) subunits in susceptible A/J strain and the more resistant 129 strain mice. The data represent the protein expression of the α3, α5, and α7 nAChR subunits in untreated A/J and 129 mice. Results represent the mean ± SD for 7 mice per strain; $^*P < 0.05$ as compared with the A/J strain.

differences in susceptibility were simply due to differences in their ability to eliminate MLA (i.e., whether the more resistant strain could more readily eliminate MLA). However, the significant differences in toxicokinetic variables observed did not explain the differences in susceptibility. For example, the $t_{1/2}$ of MLA in the brain was found to be shorter in the susceptible A/J strain, indicating the MLA was cleared more readily from the brain of A/J mice than from that of 129 mice. Additionally, the A/J mice had less bioavailable MLA in brain tissue than the 129 mice, as indicated by the differences in AUC, 0.7 ± 0.0 and 1.3 ± 0.2, respectively. Another important finding from the toxicokinetic analyses was that no differences were identified for any of the toxicokinetic variables for muscle tissue. Therefore, the toxicokinetic data indicated that the differences in susceptibility of the A/J and 129 strains of mice to larkspur alkaloid toxicosis could not be attributed to differences in the concentration of MLA in the brain and muscle tissues, but that the differences must have arisen from their different abilities to respond physiologically to the MLA exposure.

Research has demonstrated that the physiological effects of MLA are attributable to its increased affinity to nAChR in muscle, as well as in the central and autonomic nervous systems (Benn and Jacyno, 1983; Stegelmeier et al., 1998). Methyllycaconitine is a potent and selective competitive antagonist with nanomolar affinity at α7 nAChR and micromolar affinity at other nAChR (Ward et al., 1990; Alkondon et al., 1992; Lopez et al., 1998; Daly, 2005). Additionally, it has been well documented in mice that differences in the expression of nAChR subunit genes in different mouse strains are correlated with functional changes in the response to nAChR agonists (Gahring and Rogers, 2008; Rogers et al., 2008). Therefore, we compared the protein expression of various nAChR subunits in brain tissue by using immunoblotting techniques. We found that the resistant 129 strain had greater amounts of α3, α5, and α7 nAChR subunit protein expression than the A/J strain. Of particular interest was the finding that the α7 nAChR subunit expression was approximately 2-fold greater in the 129 strain. These data suggest the presence of an α7 nAChR reserve in the 129 strain, and it is likely that this reserve contributes to the resistance of the 129 strain to MLA toxicity. With a larger number of nicotinic receptors, the 129 strain would be able to tolerate a larger dose of MLA before the percentage of blocked receptors would reach a toxic threshold. The 129 strain had approximately 2-fold greater α7 nAChR, and likewise had approximately a 2-fold greater LD_{50}, than the A/J strain. These data suggest that the differences in susceptibility between these 2 strains could be due to the difference in the number of nAChR subunits. Because the tissues used for the immunoblot analyses were obtained from naïve mice, we can conclude that the differences in nAChR subunit protein expression were a constitutive difference and not a difference in response to toxicosis. Further research is needed to determine whether genetic differences are also respon-

sible for differing susceptibility, such as differences in promoters or epigenetic differences that would regulate nAChR protein expression. Additional factors that may affect the physiological response of animals to larkspur alkaloids should also be investigated.

In conclusion, the results of this study confirm previous reports that there is fairly large animal-to-animal variability in response to toxicity of the larkspur alkaloid MLA, and that this variation is found across numerous strains of mice. In addition to differences in the protein expression of nAChR subunits, other potential differences between these 2 strains will be evaluated in the future by genomic technologies. Both the protein expression differences and the potential identification of genetic markers discovered in mice will provide the basis for future experiments to identify genetic factors that correlate with susceptibility to larkspur toxicity in cattle. This research will provide livestock producers with specific information that will be useful in breeding, culling, and grazing management programs to reduce or prevent larkspur poisoning on rangelands.

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